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Original Articles

Guidelines for the Diagnosis and Monitoring of Paroxysmal Nocturnal Hemoglobinuria and Related Disorders by Flow Cytometry

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Background: Paroxysmal nocturnal hemoglobinuria (PNH) is a rare hematopoietic stem cell disorder characterized by a somatic mutation in the *PIGA* gene, leading to a deficiency of proteins linked to the cell membrane via glycophosphatidylinositol (GPI) anchors. While flow cytometry is the method of choice for identifying cells deficient in GPI-linked proteins and is, therefore, necessary for the diagnosis of PNH, to date there has not been an attempt to standardize the methodology used to identify these cells.

Methods: In this document, we present a consensus effort that describes flow cytometric procedures for detecting PNH cells.

Results: We discuss clinical indications and offer recommendations on data interpretation and reporting but mostly focus on analytical procedures important for analysis. We distinguish between routine analysis (defined as identifying an abnormal population of 1% or more) and high-sensitivity analysis (in which as few as 0.01% PNH cells are detected). Antibody panels and gating strategies necessary for both procedures are presented in detail. We discuss methods for assessing PNH populations in both white blood cells and red blood cells and the relative advantages of measuring each. We present steps needed to validate the more elaborate high-sensitivity techniques, including the need for careful titration of reagents and determination of background rates in normal populations, and discuss technical pitfalls that might affect interpretation.

Conclusions: This document should both enable laboratories interested in beginning PNH testing to establish a valid procedure and allow experienced laboratories to improve their techniques. © 2010 Clinical Cytometry Society

Key terms: flow cytometry; paroxysmal nocturnal hemoglobinuria; practice guidelines

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Background

Paroxysmal nocturnal hemoglobinuria (PNH), a rare hematopoietic stem cell disorder, has been recognized as a distinct clinical condition since the early 1800s (1,2). PNH has three distinctive clinical features that vary greatly from patient to patient and during the course of the disease (3–5). First, there is complement-mediated and predominantly intravascular hemolysis that gives rise to many of the clinical manifestations of the disease including: dysphagia, lethargy, erectile dysfunction, chronic renal failure, pulmonary hypertension, anemia, and, of course, hemoglobinuria. Second, there is a characteristic thrombotic tendency that can be life-threatening and occurs not only in the extremities but also in unusual anatomical locations, such as hepatic portal (Budd-Chiari Syndrome), splenic, or mesenteric veins. Third, there is underlying bone marrow failure, which occurs to some degree in all patients, and in its most extreme form, presents as immune-mediated severe aplastic anemia.

The disease has an estimated incidence of only 1.3 new cases per one million individuals per year. Although it has long been classified as an acquired hemolytic anemia, it is now known to affect all lineages of blood cells and is, therefore, recognized as a stem cell disorder. Thus, PNH is better defined as an acquired hematopoietic stem cell disorder in which somatic mutation of the X-linked *PIGA* gene results in partial or absolute deficiency of all proteins normally linked to the cell membrane by a glycophosphatidylinositol (GPI) anchor. PNH stem cells are thought to escape the immune-mediated destruction that eliminates normal bone marrow stem cells, and thereby become the major source of blood cell production in patients with PNH. Thus, the defining phenotypic feature of PNH cells is their deficiency of proteins that require a GPI anchor for attachment to the cell membrane. Conventionally, cells with such a deficiency are referred to as "PNH clones," and that term will be used here. However, such cells, especially in low numbers, may be neither truly clonal nor by themselves diagnostic of PNH.

Deficiency in the GPI-anchored membrane proteins CD55 (decay accelerating factor), which prevents formation and augments instability of the C3 convertases in the complement cascade, and CD59 (membrane inhibitor of reactive lysis), which inhibits assembly of the membrane attack complex of complement, are responsible for complement sensitivity of red cells in PNH. This results in the chronic and acute episodes of intravascular hemolysis that are typical of the disease. In some instances of PNH, the deficiency of GPI antigens is only partial, as the underlying genetic mutation in the *PIGA* gene allows for some synthesis of GPI anchor (6).

We now understand many of the aspects of the natural history and pathobiology of PNH and its relationship with bone marrow failure, though our understanding of the pathophysiologic mechanism which produces bone marrow failure is not as clear as that of hemolysis. However, screening for PNH and diagnosis of patients with PNH have improved considerably since the landmark discoveries relating to the underlying molecular genetic abnormality and elucidation of the biochemical defect of the disease (7,8). Although PNH is rare, screening of appropriate patients and correct diagnosis are important, because PNH is a chronic disease that persists for many years and has a profound impact on quality of life and survival for any individual patient. In the last few years, the development (9) and successful clinical trial (10) of a humanized monoclonal antibody against the terminal complement protein C5 (Eculizumab; Alexion Pharmaceuticals, Cheshire, CT) has improved the quality of life for patients with hemolytic PNH by reducing hemolysis, thrombosis, and transfusion requirements (10–13). This has made screening and accurate diagnosis of PNH important priorities for many clinical laboratories.

A classification scheme for PNH has been proposed by the International PNH Interest Group (I-PIG) that includes three main categories that cover the spectrum of disease presentation (14): (1) Classical PNH, which includes hemolytic and thrombotic patients; (2) PNH in the context of other primary disorders, such as aplastic anemia or myelodysplastic syndrome; and (3) Subclinical PNH, in which patients have small PNH clones but no clinical or laboratory evidence of hemolysis or thrombosis. The overall purpose of such a classification scheme is to provide a common international terminology for the disorder.

Diagnosis of PNH

Because PNH was initially recognized as a type of hemolytic anemia, the initial focus on red blood cells (RBCs) led to the development of several RBC-based assays. These included the Ham test and the sucrose hemolysis test, both of which demonstrated the increased sensitivity of PNH RBCs to complement-

mediated hemolysis under standard conditions (15–18). Neither of these tests was specific, and both were cumbersome to perform. A more specific test, the complement lysis sensitivity test, measured the amount of hemolysis at varying concentrations of complement; this assay showed that PNH cells lysed at lower concentrations than did normal cells (19). This test also led to the recognition that some PNH patients have a population of cells with intermediate complement sensitivity (Type II), between normal RBCs (Type I) and the most abnormal PNH-type RBCs (Type III) (20,21). However, this test is laborious, difficult to standardize and may miss small populations of abnormal cells (22).

Today, flow cytometry to detect populations of GPI anchor–deficient cells is firmly established as the method of choice for diagnosis and monitoring PNH, though there is little or no clear consensus on what represents the best approach (23,24). Compounding the problem are recent technological developments in clinical flow cytometric instrumentation. Modern digital clinical flow cytometers are now capable of analyzing at least six fluorescence parameters or antigens on a routine basis, and collecting up to one million events very rapidly. Although this increased level of sophistication and data complexity means that small populations of abnormal cells can easily be detected, in many instances, clinical significance has not yet been fully explored.

Procedures for detecting RBC and white blood cell (WBC) PNH populations are discussed in detail below. Historically, there has not been uniformity in selection of monoclonal antibodies among laboratories. This can largely be attributed to the fact that there are monoclonal antibodies available against many different GPI-anchored proteins, all of which have some capability of detecting PNH clones, especially in frank PNH where such cells are numerous. However, there are emerging data from external quality assurance (EQA) programs that indicate that some reagents are inferior to others, at least with respect to their ability to detect GPI-anchored antigens on stabilized cells. For example, although CD55 and CD59 are widely used for detecting granulocyte PNH clones, there are data to suggest that these reagents may not perform as well as antibodies to other antigens in PNH testing (24–26). Moreover, the increasing use of flow cytometry to detect small clones has magnified differences among reagents, as some reagents suitable for detecting large, obvious clones perform less well in higher sensitivity analysis.

There are emerging data to suggest that one of best reagents available to study GPI-linked antigens on leukocytes is the reagent fluorescent aerolysin or FLAER (Pinewood Scientific Services, Victoria, BC, Canada). This is a fluorochrome-conjugated inactive variant of the bacterially derived channel-forming protein aerolysin, which binds specifically to GPI-anchors. FLAER may offer significant advantages as a reagent for PNH testing; in contrast with antibodies against many of the GPI-linked antigens normally studied (25,27,28), its binding is less sensitive to the maturational stage of the cells. FLAER can also be used in multicolor combinations with monoclonal antibodies to GPI-linked and non-GPI antigens for the detection of PNH clones (29).

The Need for a Consensus Guideline for Immunophenotyping of PNH Cells

The correct diagnosis of PNH is essential for effective patient management. However, because the disease is rare and may only be tested for infrequently by many laboratories, approaches to the detection of PNH clones vary significantly (22,29–32). While there are not comprehensive data about the sources of variability, recent EQA data have highlighted a number of laboratories that were unable to detect PNH clones in known patient samples, or found PNH clones in normal samples (24).

To address these important issues, a workshop on PNH testing was held at the 2008 Clinical Cytometry Society Annual Scientific Meeting in Portland, Oregon. Most of the participants at that meeting were strongly in favor of developing a consensus guideline for PNH testing, and this document is a response to that conclusion. The document was collectively written by the authors, and went through many drafts in which several points were debated; unanimity was not reached in all cases, though on those few points that there was not unanimity, at least six of the eight authors concurred. In addition, the document was circulated to all the attendees of the workshop for their comments, and many of these were also incorporated into the document.

The challenge the authors faced in constructing this document was that, as mentioned above, many different laboratories had independently evolved procedures for PNH testing, and there were limited published data on head-to-head comparisons of these methods. Thus, although ideal recommendations in a consensus document would be evidence-based, the literature to date cannot be interpreted to mandate a single approach for all laboratories to follow to detect PNH populations. However, between the literature and the authors' unpublished collective experience, it is clear that some procedures or reagents are better than others, and these form the basis of many of our recommendations. Furthermore, some assay characteristics have not been investigated in great detail; thus some of the recommendations will simply reflect things that the authors know will work, rather than things that have proved to be necessary, or even optimal. We fully expect there to be revisions to this document as more investigation proceeds; near the end of the document, we highlight the places where more specific investigation would be helpful. While there is some variability in the approaches presented, we attempt to emphasize the general principles that form the basis of all good assays.

The document separately discusses methodology used in routine analysis from that used in high sensitivity analysis. We define the former as having a sensitivity of 1%; this is suitable for use as a screening test to detect patients with large clones typically associated with hemolytic and/or thrombotic PNH, and can also detect

many smaller clones in patients with aplastic anemia and subclinical PNH. Such assays are relatively straightforward, and many have been described in the literature; the guidelines constitute a relatively noncontroversial compendium of published practices, and assays used in the laboratories of the authors. Any laboratory that performs PNH testing should find them relatively easy to follow.

Higher sensitivity assays, defined here as assays capable of detecting clones as small as 0.01% (or even less), present a different challenge. While clinicians who take care of patients with PNH and related disorders have recommended "high sensitivity" monitoring for certain groups of patients (14), literature on the performance characteristics of such tests is scant. Achieving consensus on these assays, particularly those involving RBCs, was difficult, as the authors have varying degrees of experience with them. All agree that high sensitivity assays are more difficult than routine ones, and require special precautions. This document attempts to spell out procedures laboratories should take before offering such tests, but also illustrates results obtained when careful analysis is done.

Although the focus of the document is on analytical procedures, we also review clinical recommendations, discuss pitfalls in interpretation, and set guidelines for reporting results in an unambiguous format that can be universally understood by clinicians. We hope that this guideline will assist laboratories wanting to establish routine PNH testing for the first time, help more experienced laboratories who are considering instituting high sensitivity testing, and allow expert centers to share their experience in troubleshooting problems with lesser experienced laboratories. The widespread acceptance of a guideline may also have secondary benefits in improving the quality of information in the global PNH registry (http://www.pnhsource.com/PNH%20Registry/default.aspx) in which flow cytometric data play such a crucial component in monitoring the natural history of PNH patients.

CLINICAL INDICATIONS FOR PNH TESTING

Patients with PNH may present with a wide range of signs and symptoms, many of which are common, so that given the rarity of the disease screening every patient with anemia or thrombosis is not appropriate. However, some clinical presentations sufficiently raise the probability of finding PNH clones that they demand investigation.

Although only a minority of patients with PNH presents with hemoglobinuria, any patient with unexplained hemoglobinuria should be tested for PNH. Somewhat more controversial is the need to test patients with evidence of hemolysis; although patients with antibody-mediated Coombs-positive hemolytic anemia do not require testing in the absence of other indications, routine PNH screening may be appropriate for all patients with Coombs-negative hemolytic anemia, particularly if characteristic cellular abnormalities (spherocytes, sickled cells, schistocytes, etc) are not present, and there is no obvious infectious cause of the hemolysis. This is particularly true of patients with associated iron deficiency, as the chronic intravascular hemolysis of PNH patients leads to urinary iron loss.

Although thrombosis is a common complication of PNH, occurring in 40% of patients (12), patients with PNH uncommonly present this way; thrombosis or embolization, accounted for about 5% of patients in one series (33). However, PNH patients are more likely to have thrombotic involvement at unusual sites, including presentations such as Budd-Chiari syndrome, or cerebral thrombosis; thus unusual presentations of thrombi should warrant PNH testing. Testing is also recommended in patients with coexistent thrombosis and intravascular hemolysis or cytopenias. Approximately 10% of patients may present with abdominal pain or dysphagia; again these are nonspecific findings and routine PNH testing is not warranted unless there is concomitant evidence of intravascular hemolysis or other causes of the symptoms have been excluded.

Although absence of hemolysis essentially excludes classical PNH, other forms of PNH by definition do not have hemolysis, so that it is not appropriate to limit PNH testing only to those patients with hemolysis. How aggressively patients with cytopenias should be investigated for PNH is a matter of some controversy. Certainly, any young person with cytopenias, in whom aplastic or hypoplastic anemia is in the differential diagnosis should be screened for the presence of PNH clones, but isolated anemia rarely requires PNH testing unless an extensive workup fails to demonstrate an explanation for the anemia. A recent recommendation by the I-PIG suggested that all patients with aplastic anemia, or the myelodysplastic disorder refractory anemia (now refractory cytopenia with unilineage dysplasia (RCUD) according to the current WHO classification) should be screened yearly using "high sensitivity" assays, though the specific threshold for sensitivity was not specified (14). They further recommended that patients with other forms of MDS or with myeloproliferative neoplasms without evidence of intravascular hemolysis should not be screened outside of a research setting. Recently, however, a comprehensive study of patients with other forms of MDS has been done [the "EXPLORE" study (Examination of PNH by level of CD59 on red and white blood cells in bone marrow failure syndromes)] in which a significant number of such patients were found to have PNH populations (34). Thus, the recommendation to exclude MDS patients from routine testing may not be appropriate. A summary of the clinical scenarios for which PNH testing is recommended is given in Table 1.

Follow-up Testing

Patients with established diagnoses of PNH should have their PNH clone size monitored at regular intervals. If the disease is stable, annual monitoring may be sufficient, but any change in clinical or hematologic parameters requires more frequent monitoring; this is true whether these

Table 1
Clinical Indications for PNH Testing

Intravascular hemolysis as evidenced by hemoglobinuria or elevated plasma hemoglobin
Evidence of unexplained hemolysis with accompanying:
Iron-deficiency, OR
Abdominal pain or esophageal spasm, OR
Thrombosis (see below), OR
Granulocytopenia and/or thrombocytopenia
Other acquired Coombs'-negative, non-schistocytic, non-infectious hemolytic anemia
Thrombosis with unusual features:
Unusual sites
Hepatic veins (Budd-Chiari syndrome)
Other intra-abdominal veins (portal, splenic, splanchnic)
Cerebral sinuses
Dermal veins
With signs of accompanying hemolytic anemia (see above)
With unexplained cytopenia
Evidence of bone marrow failure:
Suspected or proven aplastic or hypoplastic anemia
Refractory cytopenia with unilineage dysplasia
Other cytopenias of unknown etiology after adequate workup

show worsening or improvement of disease, as changes in clone size in either direction may reflect the changing clinical picture. Regular monitoring is useful in patients receiving eculizumab therapy, though there is not yet consensus as to the appropriate frequency of monitoring. In the early phases of the therapy, it is useful to monitor frequently to demonstrate stabilization of the red cell clone, but once this has occurred, monitoring can be tailored to the clinical situation.

Patients presenting with thrombosis or hemolysis in whom a diagnosis of classical PNH is suspected need not be tested serially if an initial satisfactorily performed test fails to reveal a PNH clone.

For patients with aplastic anemia in whom small clones are found, serial monitoring is important because patients may progress from aplastic anemia to hemolytic PNH, and this may be presaged by an increase in the clone size. While patients with RCUD have not been reported to progress to PNH, the difficulty of distinguishing this disorder from aplastic anemia suggests that monitoring for clonal progression might also be warranted in that disease as well. If a patient with a higher grade MDS or a myeloproliferative neoplasm is found to have a PNH clone, the value of continued monitoring outside the research setting has not been established; such patients rarely, if ever, progress to PNH.

FLOW CYTOMETRIC TESTING FOR PNH

Specimen Considerations

The preferred specimen for PNH testing is peripheral blood. EDTA is the most widely used anticoagulant, and performs well, but heparin and ACD have also been used and found to be acceptable. Bone marrow should not be used outside the research setting because results are often more difficult to interpret because of differentiation-associated changes in expression of some GPI-linked proteins used for assessment in both RBC and WBC maturation. Moreover, PNH testing in patients with established MDS may also be difficult to interpret (26) as these patients may have MDS-related abnormalities in expression of some GPI-anchored proteins, most notably CD16.

Specimen transport is not a major issue in detection of PNH cells as these are stable over the usual times needed for transportation to the laboratory. RBC analysis has been successfully performed on samples kept refrigerated up to 7 days, though it is still recommended that RBC testing be performed promptly, preferably within 48 h of collection, because there are only limited data that address the question of whether PNH cells are preferentially lost over time. However, for detection of PNH on leukocytes, alterations in scatter and antigen expression of granulocyte populations that occur over time can make interpretation of results difficult; the experience of the authors indicates that satisfactory results can be obtained for samples that are processed within 24–48 h after acquisition. Some general principles about specimen handling and analysis are presented in Table 2.

RBC Analysis—Routine Assays

Introduction. As noted above, RBC antigen assays were the first used to detect PNH because DAF (CD55) and MIRL (CD59) were recognized early on as the proteins whose deficiency was central to the pathophysiology of PNH. CD58 is also a GPI-anchored red cell antigen but this occurs in both a transmembrane as well as a GPI anchored form so that it may be difficult to interpret results (35,36), and there are no data to indicate that it provides any advantage over CD59 or CD55. The goal of RBC analysis is to reliably identify and quantify cells lacking expression of GPI-anchored proteins (Type III cells), and to distinguish them from normal RBCs (Type I cells). RBC analysis should also recognize and quantify cells that are partially deficient (Type II cells) if they are present (Fig. 1). Testing of RBCs alone in a routine assay is not adequate for evaluation of PNH patients, because hemolysis and transfusion may greatly underestimate the size of the PNH clone (37). For these reasons, WBC clones are frequently detected when RBC clones are not, though significant RBC clones are never seen without WBC clones (37). Nonetheless, RBC testing is still important, both because Type II cells are more readily detected, and because comparison of the relative sizes of RBC and WBC clones may provide useful clinical information.

Sample processing. RBC assays for PNH differ from WBC-based flow cytometric immunophenotyping assays in lacking a RBC lysis step, and in including strategies to prevent RBC agglutination. A procedure using cell staining with antibodies followed by washing is recommended. Washing the RBCs more than once can help to decrease nonspecific binding of antibody and/or remove excess fluorochrome that may make distinction between

Table 2
Sample Considerations

Sample source	Peripheral blood (bone marrow not optimal)
Anticoagulant	EDTA, heparin, or ACD
Preferred sample volume	Minimum 1 ml; 3 ml adequate for most testing though more possibly needed if WBC very low
Maximum sample age	Up to 7 days for RBC; <48 h for WBC
Sample storage	4 degrees after 24 h
Lysing reagent	For WBC, commercial lysing reagents have not been rigorously studied but no commercial reagent is known not to work; ammonium chloride a satisfactory alternative
	No lysing for RBC
Sensitivity in routine analysis	1%; at least 5,000 events of specific cell type collected
High-sensitivity analysis	0.01%; at least 250,000 events of specific cell type collected
Cell populations analyzed in routine analysis	Granulocytes in all cases; Monocytes provide confirmatory information.
	RBC in AT LEAST those cases with a PNH clone detected by WBC analysis, or in all cases.
	Routine analysis of RBCs alone not recommended. No role for analysis of lymphocytes.

Type III and Type II cells difficult (see Fig. 2). Washing may also help decrease aggregation, as may vigorous vortexing or other types of mechanical disruption, which might include something as simple as running a tube with a resuspended pellet across a test tube rack, to disaggregating cells through a narrow bore pipette prior to acquisition on the flow cytometer. It is also critically important to avoid IgM antibodies, and to dilute all antibodies appropriately, as high concentrations of anti-RBC antibodies promote aggregation (see below).

Selection of antibodies. In most cases of straightforward PNH, a clone can readily be detected with a single marker, though it should be noted that rare cases of congenital deficiency of CD55 or CD59 have been reported (38,39). Because CD59 is expressed at a high level it can be adequately detected with a variety of fluorochrome-conjugated antibodies. Note, however, that some CD59 clones delineate Type III from Type II cells better than others. Indirect staining is not optimal. CD55 is less abundantly expressed on red cells; it is not recommended as a sole reagent because it frequently does not provide adequate separation to identify Type II cells (see Fig. 3). How much if anything it adds to routine analysis is a matter of some controversy, but if it is used, it is preferable to use a phycoerythrin (PE)-conjugated antibody. Some investigators who use it prefer to evaluate CD55 and CD59 in different colors in one assay tube to detect populations of deficient cells using multicolor dot plots, in a similar fashion to the flow cytometric evaluation of WBCs. Some assays combine more than one anti-GPI antibody labeled with the same fluorochrome to increase the intensity of fluorochrome emitted light, but given the generally adequate separation between GPI-anchor deficient RBC clones and normal RBCs with CD59, this approach may not be necessary. Strategies that use both CD55 and CD59 increase the need for careful titration of antibodies to avoid RBC agglutination.

Red cell agglutination decreases substantially when red cell antibodies are diluted from the manufacturers' recommended concentrations, so that it is desirable to use the lowest concentration of anti-red cell antibodies that maintains an acceptable ratio of positive to negative fluorescence, with clear separation of populations. Gentle (to avoid aerosols) but thorough mixing is essential to ensure uniform staining, especially if small volumes of reagent are used.

Acquisition, gating, and analysis. In routine analysis, RBCs can be identified by their light scatter properties; log/log displays of forward and side scatter are superior for identifying the RBC population because it is easier to exclude debris, especially platelet debris, on such plots, and significant aggregation, if present, can be more readily assessed. Collection of as few as 5,000 RBCs is generally sufficient to detect populations representing at least 1% of cells. The addition of antibodies to glycophorin A (CD235a), a mucin-like transmembrane protein, allows distinction of RBCs from cells of other lineages and debris, which may otherwise be misinterpreted as deficient RBCs; in addition, it provides a useful positive control and can ensure that red cells pipetted into an antibody-containing tube have been adequately mixed. However, the surface density of glycophorin A on RBCs is so high that employing anti-glycophorin A conjugates at saturating concentrations will result in significant aggregation of red cells (37). Therefore, careful titration of anti-glycophorin A antibodies needs to be performed to minimize this. In general much higher dilutions than those used for bone marrow immunophenotyping will likely be required. Aggregation is typically greater with PE (or PE tandem–based) conjugates than with FITC conjugates of anti-glycophorin A. Because information on protein concentration and fluorescence/protein ratio is generally not readily available and may vary among manufacturers and between lots, it is not possible to stipulate a specific protein concentration to use; it is important to stress that each laboratory should carefully evaluate the chosen clone and conjugate to determine the optimum concentration that both limits aggregates and provides an acceptable signal on positives.

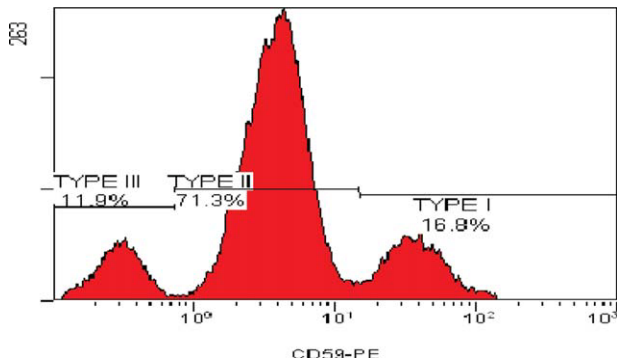

FIG. 1. CD59 (clone MEM-43 from Invitrogen) expression on RBCs in a patient with PNH showing separation of Types I, II, and III cells.

The identification of Type II cells has not been standardized. To avoid issues related to variation of expression between samples, it is preferable to compare staining of a putative Type II population with any Type I cells present within the same sample, (36,40) and, as noted above, to ensure that such cells do not represent Type III cells with incomplete washing. It is important to remember that transfused cells may differ slightly in level of expression of GPI-linked proteins from native RBCs and therefore may appear as a separate, overlapping population. A negative control consisting of unstained cells is often useful for identifying the expected position of Type III cells; if such a sample is mixed with an appropriately stained normal sample, the expected position of Type II cells can be identified as the area between the positive and negative populations.

PNH clones can be recognized either with single-color histograms or dot plots. Single-color histograms facilitate comparison of the level of RBC staining with that expected for normal and deficient cells, and are preferable for the separate identification and quantification of Type II and Type III cells when they represent enough of the total events to form a separate peak (see Fig. 1). Dot plots provide the most accurate method for quantifying abnormal populations when they represent a small proportion of total cells (Fig. 4). When multicolor analysis is used, they are the best method for demonstrating populations of cells deficient in more than one antigen, but even in single color analysis dot plots of scatter vs. CD59 may be useful for identifying small populations.

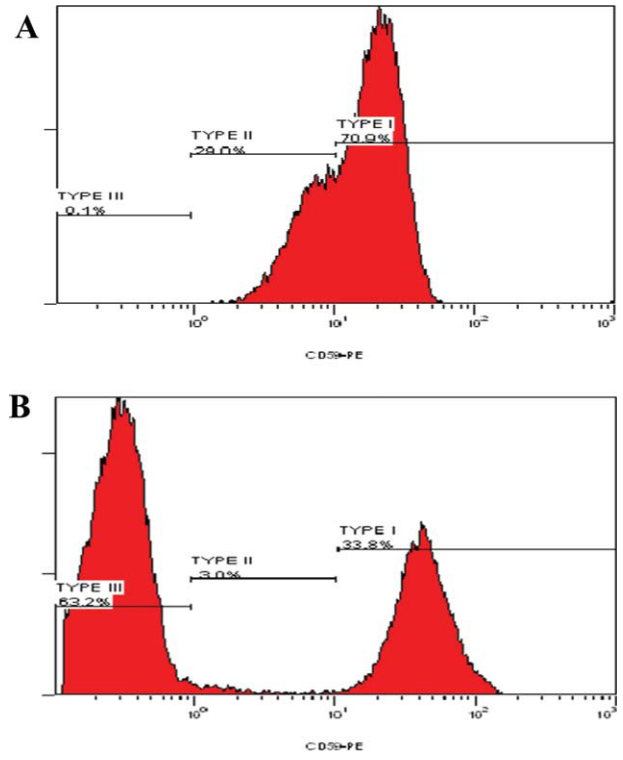

FIG. 2. Effect of washing on CD59 (Invitrogen, clone MEM-43) staining of PNH red cells. In the absence of washing (**A**), either excess free PE fluorochrome or non-specific binding demonstrates only a shoulder suggestive of a Type II population, and no definite Type III population. Appropriate washing (**B**) shows that these are in fact Type III cells.

Leukocyte Assays—Routine Analysis

Introduction. Assessment of PNH populations in leukocytes is widely recognized as the best method for assessing the true size of a PNH clone. Lymphocytes are not a suitable target because of their long life span and variable expression of many GPI-linked proteins. However, both monocytes and neutrophils are suitable targets; generally speaking the clone size measured in each population agrees relatively closely, and though independent assessment of both cell types is not absolutely necessary, it is relatively simple to do with most panels used. Moreover, the assurance gained by detecting the abnormality in both populations adds to the confidence in diagnosis.

Sample processing. Most assays of GPI-linked antigen expression on leukocytes are performed with a stain-then-lyse procedure because light scatter characteristics useful for gating are generally better preserved than with samples that are prelysed and then stained. Although there are no data comprehensively comparing

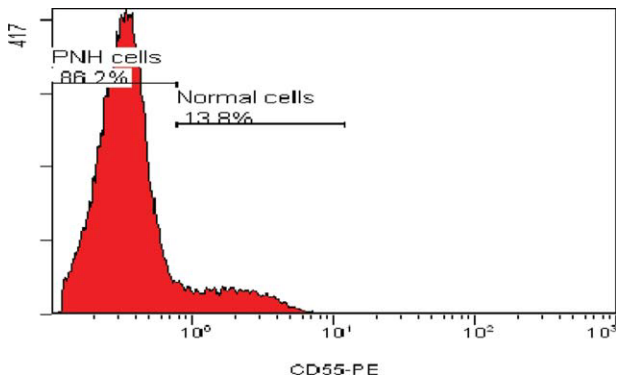

FIG. 3. CD55 (clone 67 from Invitrogen) expression on RBCs in the same patient illustrated in Figure 1. Although the overall percentage of PNH cells detected is similar, there is no separation of Types II and III red cells.

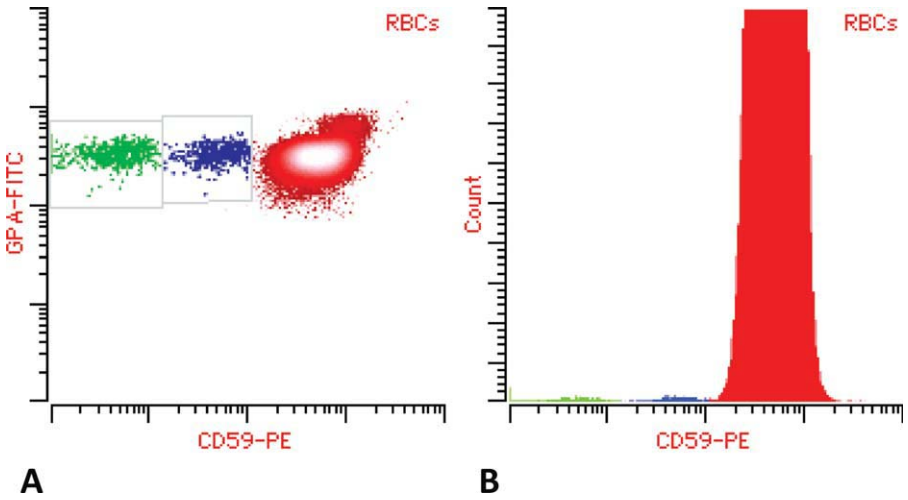

FIG. 4. Comparison of dual parameter plots and histograms for demonstrating small red cell clones. **A**: Dual parameter display of GPA(CD235a, clone KC16 from Beckman-Coulter) and CD59 (Clone MEM43 from Invitrogen) in a patient with a small red cell clone comprising a mixture of Type II (blue) and Type III (green) cells. The two populations are readily distinguished and can be easily quantified with appropriate gates. **B**: Same data displayed as a histogram. Even scaled, the large number of Type I RBCs makes it difficult to recognize the PNH red cells and makes it hard to set discriminators among the populations.

different commercially available lysing reagents, the authors are not aware of any that do not perform satisfactorily. If the leukocyte count is low, prelysing with NH4Cl may be preferable.

Selection of antibodies. Historically, CD55 and CD59 were the first markers used for detecting PNH clones in granulocytes as WBC testing grew out of prior experience with RBC testing. However, these markers generally give less separation between positive and negative populations than other GPI-linked antigens. In EQA studies, CD55 and CD59 had significantly higher coefficients of variation and yielded lower clone sizes than did either CD16 or CD66b (24). A wide variety of GPI-linked antigens have been described on granulocytes. Of these, most experience exists with CD16, CD24, and CD66b. CD16 is absent from eosinophils and may be lost from granulocytes in cases of myelodysplasia. In addition there are polymorphic variants of CD16 that are not recognized by some anti-CD16 antibodies (41) so that CD16 is usually best combined with another reagent. CD14 is a GPI-linked marker expressed on monocytes that is commonly used to detect monocyte clones, though it is absent from some immature monoctyes and dendritic cells, so its usefulness in detecting small monocyte clones is limited. Other markers, such as CD48 and CD157, could in principle be used to detect WBC clones, though there is limited experience with these reagents. CD55 (but not CD59) is expressed relatively brightly on monocytes and has been shown to be useful for identifying PNH monocytes (42).

FLAER (Pinewood Scientific, Vancouver, BC), which binds specifically to the GPI anchor and is consequently reliably absent from GPI anchor–deficient granulocytes and monocytes, has become perhaps the most useful reagent for detecting WBC PNH clones. Most early studies used a lyophilized form of the reagent (27,29), whose inconvenience of use and problems with stability limited its widespread acceptance in routine clinical laboratories; recent lots of this reagent appear to be more stable, however (Illingworth, unpublished observations). Recently, a liquid preparation of FLAER has been prepared that shares the binding characteristics of the lyophilized form, but has stability and storage requirements comparable to those of typical monoclonal antibodies. Nonetheless, it is still important to protect this reagent from light and from prolonged exposure to temperatures above 2–8° (Borowitz, unpublished observations).

Gating and analysis. Gating rarely presents a problem for assessment of loss of GPI-linked antigen expression (or of FLAER binding) on granulocytes in the typical case of PNH with large clone size. Either FSC/SSC or CD45 vs. SSC displays can identify these with reasonable assurance and an accurate gate set. Monocytes may not represent such a pure population on these displays, particularly if they are present in low numbers, so that lineage markers may be more useful for identifying them. Lineage markers can also be very useful for increasing the purity of the granulocyte gate, something of particular importance when higher sensitivity assays are utilized (see below), or in cases such as myelodysplasia in which altered light scatter of neutrophils makes gating difficult.

For routine purposes, collection of 5,000–10,000 cells of interest is generally sufficient to detect populations at a sensitivity of 1% or even better. Acquisition of this number of monocytes may be more difficult, and smaller numbers are acceptable, especially if results on granulocytes and monocytes agree. As a general rule, it is desirable to have both positive and negative populations present within the gate for accurate placement of discriminators, so that in cases with very large PNH clones it might be necessary to collect more events to demonstrate the population expressing GPI-linked antigens with certainty. For large clones, it might be possible to have a single GPI-linked marker or FLAER and discriminate positive from negative events using a single parameter histogram, but for the majority of cases, it is most desirable to use two different markers and set quadrant markers or regions that readily identify the negative

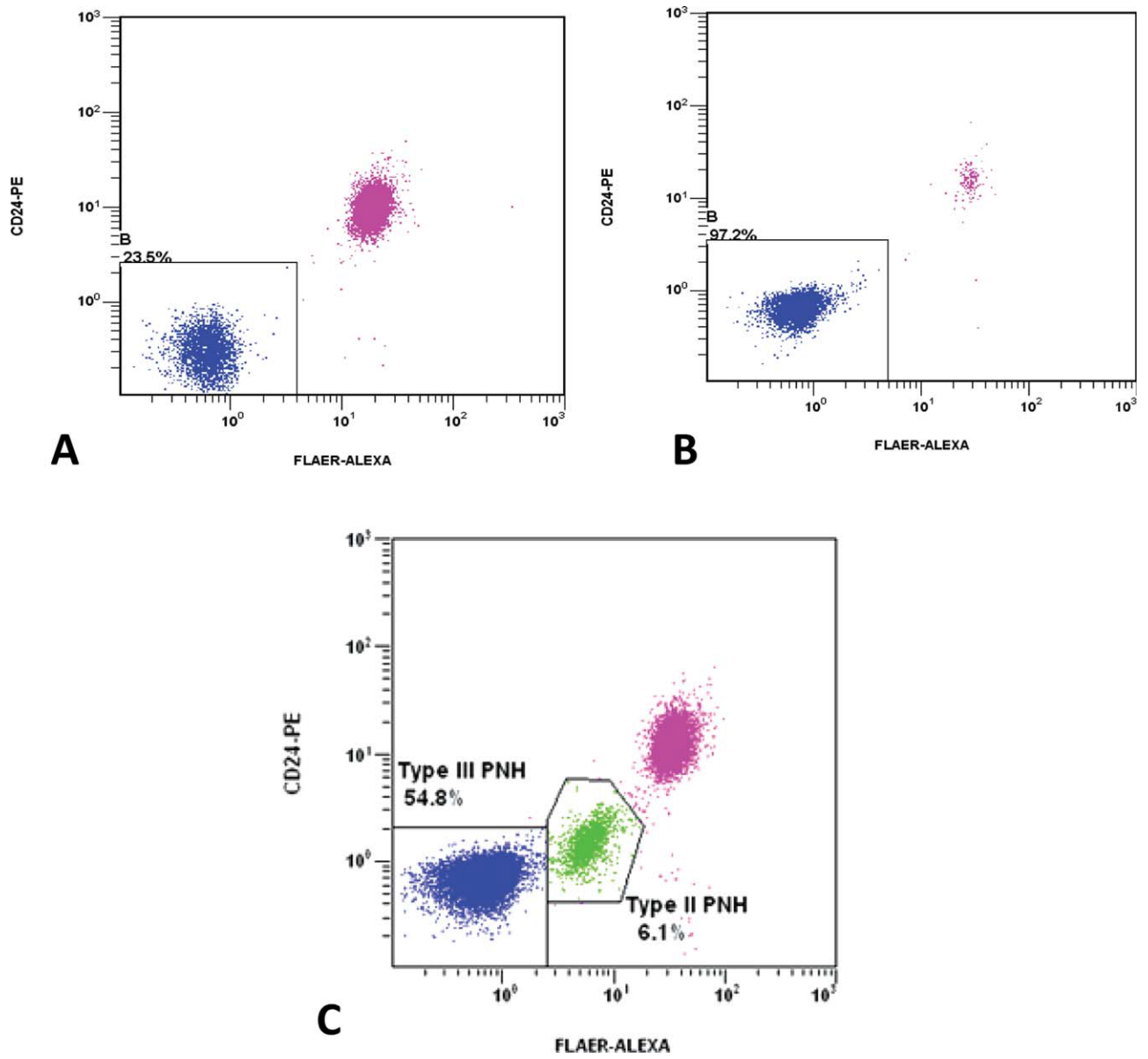

FIG. 5. Display of FLAER vs CD24 (Clone ALB9 from Beckman Coulter) in three PNH patients. **A**: PNH granulocyte population of 23.5% **B**: PNH granulocyte population of 97% **C**: PNH granulocyte population of 60.9% comprising 54.8% Type III cells and 6.1% Type II cells.

population of interest (Figs. 5a and 5b). Occasionally, a population of Type II granulocytes may be identified, especially when FLAER is used (Fig. 5c), while the clinical significance of identifying Type II granulocytes is not known, their presence should be mentioned if observed and should also result in a careful search for the possible presence of Type II RBCs. When computing the total size of the granulocyte clone, it is important to combine both the Type II and Type III granulocytes (43).

Higher Sensitivity Assays

As noted above, improvements in flow cytometry technology and the increasing use of rare-event analysis has made it possible to detect smaller and smaller abnormal populations, provided care is taken in analytical procedures. Given an adequate number of total cells analyzed, events as rare as one in 10 million have been reported (44,45). However, this level of sensitivity is rarely reached in practice, and is dependent both on the cell type analyzed and the ability of markers to distinguish the desired population. In rare-event analysis, it is critical to limit false-positive events; acquisition of sufficient events to identify a population of cells with a characteristic phenotype and evaluation of multiple parameters to identify uniquely the desired cells are essential in achieving this goal. Other challenges for

rare-event detection by flow cytometry include nonspecific staining, autofluorescence, carryover from previous samples, and erroneous data bursts (44).

High-sensitivity assays are not needed for the diagnosis of classic PNH but are much more useful for the detection of small PNH populations in patients with bone marrow failure disorders. Aplastic anemia and PNH have long been associated (46). PNH was commonly observed as a complication of aplastic anemia after effective immunosuppressive therapy (47–49). PNH cells are now known to be found in the majority of patients with aplastic anemia and in some with RCUD (50–52). Sequencing of the *PIGA* gene in some patients with even very small populations proved that these were clonal (52) More recently, PNH clones have been detected in other types of MDS (34). However, the percentage of PNH cells in these bone marrow failure disorders is typically much lower than in classic PNH, requiring more sensitive assays for detection and monitoring. The presence of PNH cells in aplastic anemia or RCUD has been shown in some studies (51,53), though not in others (54,55), to correlate with a high probability of response to immunotherapy.

Rare PNH cells have been identified in otherwise healthy people (53,56–58). In a study of nine normal individuals, CD11b selection of granulocytes combined with CD55 and CD59 staining showed an average frequency of 22 PNH-type cells per million total cells (56). *PIGA* mutations were demonstrated in six of these nine samples after sorting and polymerase chain reaction (PCR). Of note, these mutations were transient, in that these clones disappeared in subsequent samples from the same patients. Small PNH RBC populations could also be detected at a frequency of eight per million, although confirmation of genotype was not possible with RBCs. In another study, a threshold of 50 per million PNH cells was suggested as abnormal, on the basis of the mean + four standard deviations of 68 normals, although the actual mean was not revealed (53). PNH populations could also be detected in normals at very low frequency by another technique, in which normal cells were first eradicated with aerolysin, leaving only GPI anchor–deficient populations (57,58); estimates of the frequency of PNH-type cells in normals using this technique ranged from 5 to 60 per million cells, with an average of 18 per million (58). Sequencing of the *PIGA* gene in some of these cases revealed that mutations were not clonal (57), and it was suggested that these mutations might occur in a colony-forming cell rather than a hematopoietic stem cell, thereby accounting for their transient nature (59,60).

The lower limit of sensitivity for detecting PNH leukocyte populations has not been determined. By extrapolation from other applications of rare-event analysis, sensitivities of 0.01% or even better should be readily achievable. Published studies of RBC methods have suggested analytical sensitivities of at least 0.005% (53).

The main determinants of sensitivity in rare-event analysis are the number of events acquired and the ability to discriminate between positive and negative events. Poisson statistics determine the minimum error rates at a given cell count and cutoff. If one counts 200,000 cells with the goal of detecting an abnormal population of 0.005% PNH cells, then a criterion of four or more events will correctly identify 99.0% of the abnormal populations. Poisson statistics can also help design screening strategies for deciding when more cells are needed for analysis. For example, to achieve a sensitivity of 0.01%, if 30,000 events are collected and zero events lack GPI antigens, there is a 95% probability that the true frequency of GPI-deficient cells is $<0.01\%$; at 50,000 events this probability becomes $>99\%$. Thus, one could screen with lower numbers of cells and reflex to collecting more events only in cases in which one or more putative PNH cells was seen.

Although this calculation helps to establish the sensitivity of an assay, the specificity of classifying a population as abnormal (i.e., the false-positive rate) depends on the background frequency of PNH cells in normals. As noted above, estimates of this frequency have varied considerably, although a recent study of 70 normal individuals estimated a frequency of 1.7 per million (Wittwer, unpublished results). Assuming this frequency, and again counting 200,000 cells, then the same criterion of four or more events noted above will falsely identify only 0.1% of normal samples as abnormal. However, if the background frequency were 22 per million, as suggested from some other studies, then a criterion of 11 events out of 200,000 cells would be necessary to achieve the same specificity, though at this cutoff there would be potentially more false negatives when only 0.005% PNH cells were present. Poisson statistics can be used to calculate appropriate criteria and numbers of cells to collect for even higher background rates. Because technical factors may contribute to the differences in background levels reported, before employing an ultra-high sensitivity assay, it is important for each laboratory to establish the apparent PNH background in the normal population and select appropriate cell counts and cutoffs.

RBCs are useful targets for rare-event analysis in PNH assays because they are readily available in large numbers, and the density of GPI-linked antigen expression is typically high; however, the frequent presence of RBC aggregates may make accurate enumeration difficult. RBCs must first be positively selected for rare-event analysis by staining for a pan-RBC antigen. Although it is generally recommended that rare-event analysis use at least two primary identifiers in a Boolean gating strategy to accurately identify the cells of interest, glycophorin A is the only RBC-specific reagent commonly employed for their primary identification, limiting positive cell selection to one antigen and light scatter gating. Furthermore, almost anything interacting with the red cell surface can produce aggregation. As noted above, saturating amounts of antibodies against GPI-linked antigens or glycophorin are not necessary and, in fact, should not be used. Also note that the concentrations of

antibodies against CD55 or CD59 that limit aggregation best may differ depending on whether they are tested individually, together, or in combination with anti-glycophorin A.

PNH cells are usually identified by absence of staining for CD59 with or without CD55. If both are used, they may be employed either in different colors or sometimes combined with the same fluorochrome, so that only cells lacking both markers are considered negative; however, unless care is taken in titration, using both reagents may increase RBC aggregation. Dot plots are preferable to histograms to ensure that the small number of events typically seen in rare-event analysis constitutes a distinct population. Moreover, if used alone, CD59-PE is superior to CD59-FITC for distinguishing small PNH RBC populations from normal CD59-positive erythrocytes, largely because CD235a-FITC is superior to CD235a-PE; it is possible to detect fewer than 0.01% Type III PNH RBCs with such an assay (Fig. 6). However, as Figure 6 shows, there may be a limit to how well Type II cells are detected by high-sensitivity techniques; while it may be that combining CD55 and CD59 will decrease background and thereby improve detection of Type II cells, the relative advantages of using both CD55 and CD59 compared with CD59 alone in a high-resolution assay have not been rigorously studied.

Critical to high-sensitivity testing is a low rate of CD55/CD59-negative events that might falsely be interpreted as PNH populations. A major potential contributor to this problem are fragmented RBCs, though these usually can be recognized on the basis of their low glycophorin A staining. If an unstained control antibody is used to determine position of the threshold for positivity, care must be taken to ensure that there is not even minimal carryover into the test sample; in fact, if such a control is used, it is better to run this after, rather than before, the test samples, or to run a blank saline-only tube between the control and test.

For high-sensitivity analysis of leukocytes, gating on light scatter alone, or even light scatter plus CD45, is similarly not acceptable because spurious events that may not represent granulocytes might be included in the gate, leading to a false conclusion that a PNH population is present. For the most accurate gating, lineage markers, such as bright CD15 for granulocytes, or bright CD33 or CD64 for monocytes are generally employed; combining light scatter and/or CD45 expression with lineage markers will further improve the accuracy of gating (Fig. 7). As a general rule, the smaller the PNH population one is trying to detect, or the more deteriorated the sample being studied, the more critical careful, multiparameter gating is to ensure that the population being analyzed contains only the cells appropriate for the denominator. Moreover, it is essential to use more than one GPI-anchored marker to detect small populations, especially if at least one marker is also absent from any minor population that might contaminate a gate; FLAER is particularly valuable for this purpose. For example, if a granulocyte gate is contaminated with a few monocytes CD24 will be negative on these cells and might be misinterpreted as a small PNH population; combining CD24 with FLAER and requiring absence of both markers will improve accuracy. Similarly, CD14 is not acceptable as a single marker for detecting small PNH populations because even a lineage-specific monocyte gate will include dendritic cells that lack CD14; this problem is overcome by combining CD14 and FLAER. Again, determination of background rate by studying normal populations can help to establish the sensitivity level that is practical to attain with any given assay (Fig. 8).

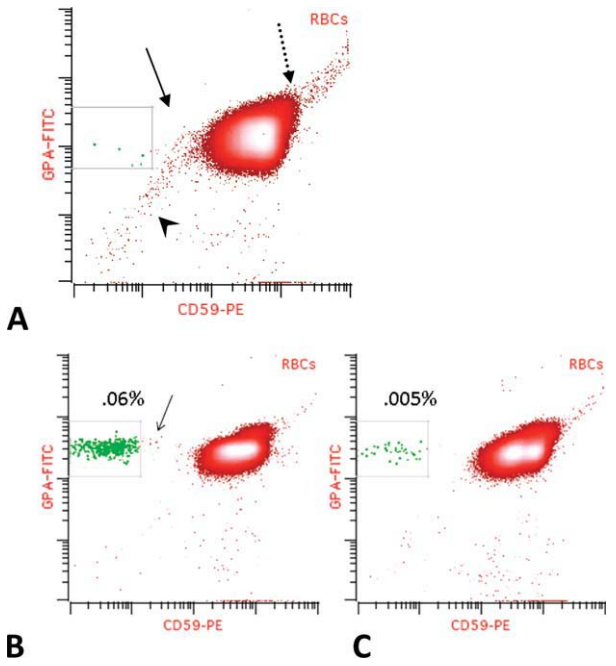

FIG. 6. **A**: High sensitivity PNH assay of a normal individual performed to determine background rates. One million cells were acquired and gated by forward scatter and side scatter, and the CD59-negative population detected on a display of CD59 vs. GPA. While gating is not optimal—setting an additional gate on scatter vs. GPA would "clean up" this display considerably—the data are deliberately displayed this way so that the various populations that must be accounted for in analysis can be identified. There are five events in the region in which Type III cells would be expected, giving a background frequency of 0.0005%. The trailing GPA-negative and CD59-negative events (arrowhead), which represent red cell fragments or incompletely stained red cells, and the cluster of brighter CD59+ and GPA+ events, which represent aggregates (dashed arrow) should theoretically be excluded from the denominator when quantifying the abnormal population, though if the assay is done properly these will be so few that they will not make a significant difference. Note that the debris-events may extend into the region in which Type II events might be expected (arrow), indicating that it is not possible to achieve as high a sensitivity for detection of Type II cells. **B** and **C**: Performance of the same assay in two individuals with very small red cell clones showing the ability to detect 0.06% (B) and even as low as 0.005% (C) Type III PNH cells. There are a few events in B that might be interpreted to represent Type II cells (arrow) but given the background findings, these cannot be classified with certainty. (Note that the GPA-positive population is slightly brighter in B and C than in A, necessitating moving the gate on the *Y* (but not the *X*) axis) (Reagents as in Fig. 4).

Table 3 summarizes gating strategies and reagent combinations used for routine vs. high-sensitivity testing. As

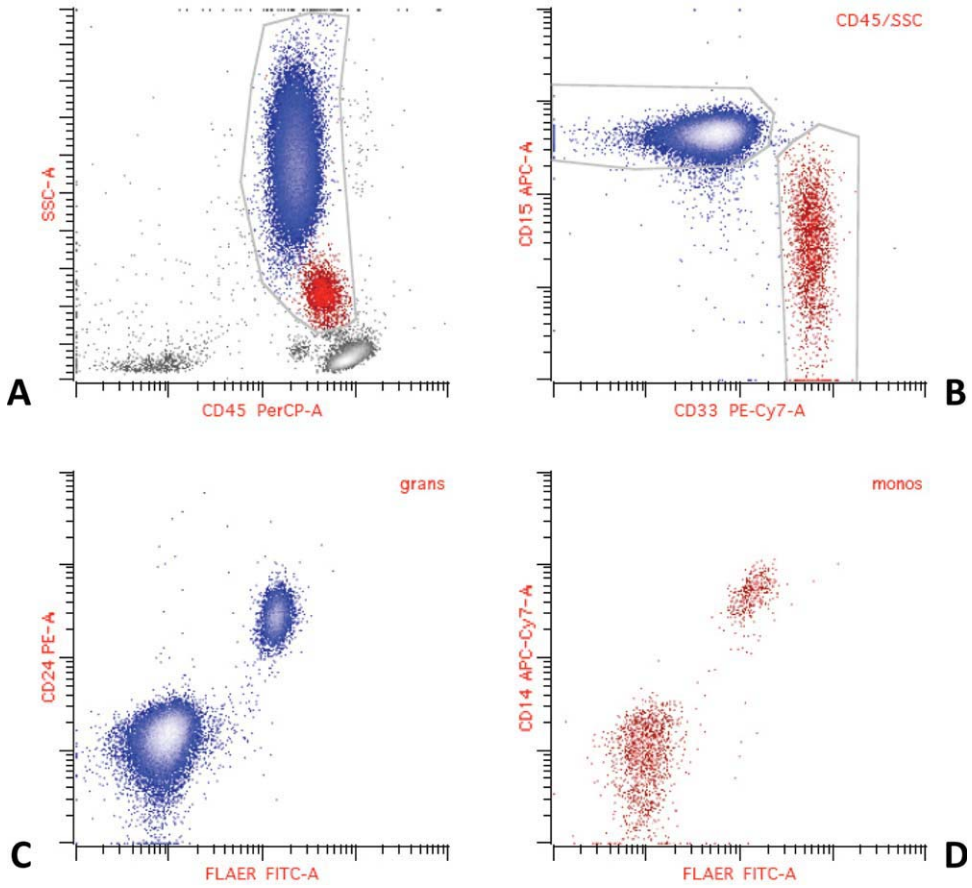

FIG. 7. A multiparameter gating strategy for granulocytes and monocytes. **A**: Initial gate set on a CD45/SSC display to include both granulocytes and monocytes. **B**: Display of CD33 vs. CD15 on the gate shown in (A). Granulocytes (CD15 high, CD33) low are clearly separated from monocytes (CD15 low CD33 high) and are easily distinguished. Setting tight gates eliminates problems with degenerating cell populations. **C**: Display of FLAER and CD24 on the CD15 positive population showing a clear double negative PNH granulocyte population. **D**: Display of FLAER and CD14 on the CD33 positive population showing a clear double negative PNH monocyte population. (CD45-PerCP clone 2D1; CD33-PeCy7 clone P67.6; CD15-APC clone W6D3; CD24-PE clone ML5; CD14-APCCy7 clone MΦP9; from BD-Biosciences).

discussed above, higher sensitivity testing requires more reagents to gate accurately, and more than one GPI-anchored probe to quantify accurately the size of PNH clones. Also, it is obvious that larger numbers of cells are required to perform high-sensitivity testing, so that as a rule, it is often difficult to perform this testing on monocytes. It recommends a limited number of reagents for WBC analysis, but does not mandate any particular combination or combinations as there is still some variability in acceptable approaches. However, many of the authors have significant experience combining CD24 and FLAER in granulocyte analysis or CD14 and FLAER in monocyte analysis, and Appendix A illustrates a number of specific three- to six-color reagent combinations based on these markers that have been shown to produce excellent results. These could provide the basis for more specific standardization of WBC methods.

Quality Control and Proficiency Testing

To date, there are no external QA data, interlaboratory or published intralaboratory studies on the reproducibility or precision of detection of very small PNH clones. For both high-sensitivity and routine analysis, familiarity with test performance and interpretation leads to greater confidence in the results. Evaluation of normal control samples and background rates is particularly important, as the purpose of the screening test is to detect antigen deficiency. In virtually all samples tested, whether they contain PNH clones or not, internal normal control populations of leukocytes should confirm reactivity of the antibodies/reagents used in the test procedure. In addition, it may be desirable to include a normal sample to ensure that under assay conditions 100% of normal cells express the antigens tested. Unstained controls may be useful for demonstrating the position of expected negative populations, but for most GPI-linked markers and FLAER, normal and deficient populations are clearly separated.

A more difficult problem is that of a positive control. PNH is a rare disease, and most laboratories do not see large numbers of samples from PNH patients, so that it is often not practical to run a positive control (i.e., a

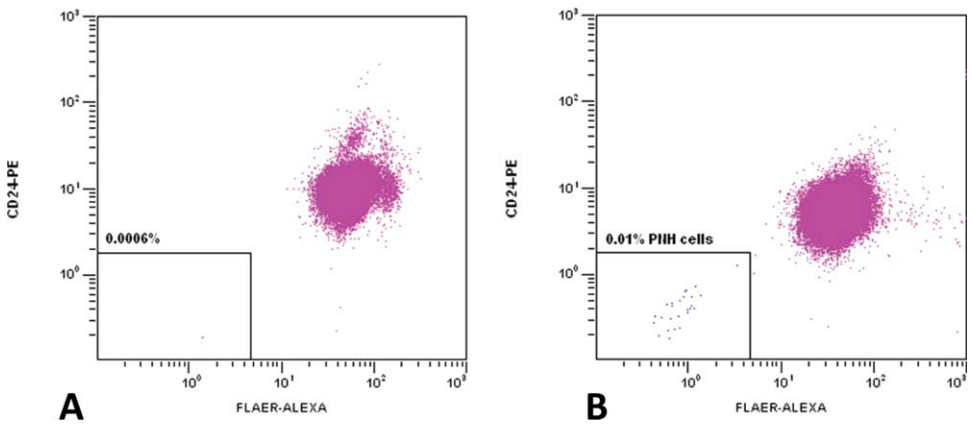

FIG. 8. High sensitivity white cell PNH assay. Granulocytes were identified on the basis of forward and side scatter, and CD45 and CD15 expression, and stained with CD24-PE (Clone ALB9 from Beckman-Coulter) and FLAER. **A**: Detection of background rate in a normal individual. 315,617 granulocytes were acquired and two CD24 and FLAER negative events were detected. **B**: Detection of a small PNH clone (56 cells/432,797 total granulocytes, 0.013%).

sample with PNH cells) with every sample. To date, no satisfactory commercial control material exists for clinical laboratories. PNH cells may be aliquoted and frozen, though this is more readily done with red cells, as PNH RBCs frozen in 20–25% sucrose/dextrose are stable indefinitely. However, laboratories must still participate in a proficiency testing program. Currently, the College of American Pathologists offers a program focused on RBCs, and has recently introduced a WBC program that is problematic because it does not work with most laboratories' gating strategies, while the UK National External Quality Assessment Schemes (UK-NEQAS) for Leucocyte Immunophenotyping has a program for both RBCs and WBCs. For laboratories reporting results from high-sensitivity analysis, interlaboratory exchange should be encouraged, because current external programs do not test these adequately. Interlaboratory exchange may also be useful for those labs that only rarely see patients with PNH.

INTERPRETATION AND REPORTING

Patients with Classic PNH

When there is a high clinical suspicion of PNH, interpretation of immunophenotyping studies that demonstrate the presence of large PNH clones is straightforward. The proportions of GPI-deficiency in the myeloid-lineage populations tested (i.e., granulocytes and monocytes) should in most instances be the same; though discrepancies have been encountered by the authors, with monocyte clones exceeding granulocyte clones in a small but notable percentage of cases; the clinical significance of this is uncertain. Even in patients with large PNH clones there is almost always a small population of residual normal cells that can be used not only to

Table 3
Antibodies Useful in PNH Testing

Type of analysis	Target cell	Gating strategies	Informative reagents
Routine	Red cells	Log FSC/SSC; glycophorin A optional	CD59 (CD55)
	Granulocytes	CD45/SSC or CD15 (or equivalent)/SSC	FLAER, CD24, CD66b, CD16[a]; two reagents preferred. CD55/CD59 combination not recommended.
	Monocytes	CD45/SSC or CD33/SSC[2] or CD64/SSC or CD163/SSC[3]	FLAER, CD14,[b] CD48,[c] CD55,[c] CD157[c]
High sensitivity	Red cells	Glycophorin A+scatter	CD59+/−CD55 in same or different colors
	Granulocytes	CD15/SSC	FLAER, CD24, CD66b, CD16[a]; two reagents essential. CD55/CD59 combination not recommended;
	Monocytes		See[d]

[a]Polymorphic variants and absence of CD16 from eosinophils may limit usefulness; should never be used as sole reagent.
[b]CD14 is negative on dendritic cells and basophils that could be included in a monocyte gate, and is also dim or negative on immature monocytes, so should not be used as sole reagent; may be useful to combine with FLAER in dual parameter analysis, though FLAER may also be dim on normal basophils.
[c]Limited experience with these reagents.
[d]Monocytes may not be suitable for high sensitivity analysis because of the difficulty in collecting sufficient events, but if performed, lineage specific gating using CD33 or CD64, and FLAER plus another reagent is essential.

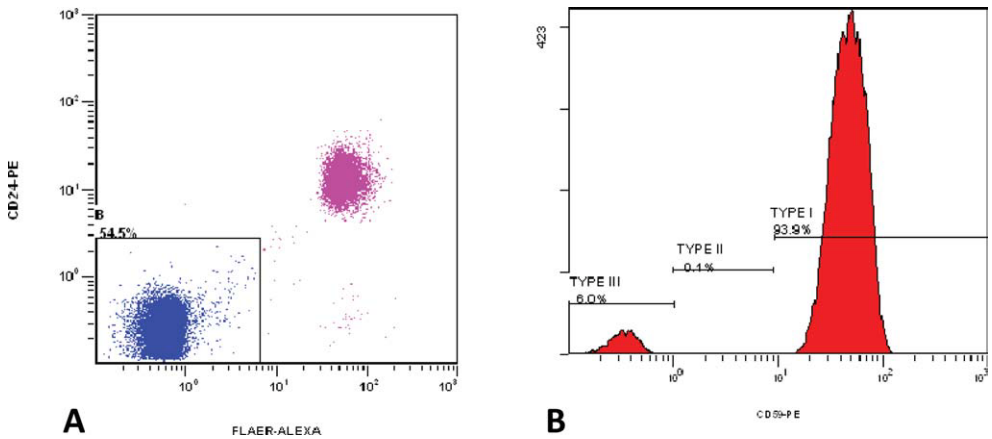

FIG. 9. Analysis of granulocytes (**A**) and red cells (**B**) in the same specimen from a patient with classic hemolytic PNH. There is a granulocyte clone of 55%, while the RBC clone size is 6%. (CD59-PE clone MEM43 from Invitrogen; CD24-PE clone ALB9 from Beckman-Coulter).

confirm antibody reactivity but also to guide positioning of analysis regions or quadrant markers. The proportions of PNH cells can be determined from standard analysis regions, or quadrant statistical analysis on gated granulocytes, neutrophils, or monocytes according to the specific combinations of antibodies used. To avoid potentially ambiguous and confusing reports, one should indicate the PNH clone percentage, not the percentage antigen expression by the residual normal cells.

RBC analysis and interpretation of CD55 or CD59 staining are often a more complex problem (23). It is important to be familiar with staining profiles for normal RBC expression of the GPI-linked antigens studied, and to know the appearance of unstained RBCs. These two reference populations can act as guides to the boundaries for discriminating Type I, II, and III cells. PNH RBCs are often present in significantly lower proportion than the corresponding granulocyte and monocyte PNH clones in any individual patient both because red blood cells have a shortened survival in the circulation whereas granulocytes do not (61) and because the patient may have been transfused and/or experienced an episode of intravascular hemolysis prior to testing (Fig. 9).

However, RBC staining does provide useful information, and is significantly better at demonstrating partial antigen deficiency than granulocyte analysis. While there is no specific cut-off that determines when PNH patients are likely to be symptomatic, patients with >20% Type III red cells are likely to show clinical signs and symptoms associated with intravascular hemolysis. In contrast, patients with large Type II populations in the absence of significant Type III populations may show a reticulocytosis and modestly elevated LDH, but less hemolysis than a patient with an equivalent number of Type III cells. The percentage of the PNH RBC population, and the proportions of Type II and Type III components should be reported. In some instances, these two PNH populations cannot be clearly distinguished, and a comment can be added to highlight this.

Monitoring of the RBC PNH clone is also useful for assessing efficacy of response to eculizumab therapy (62). Because this drug inhibits complement lysis of GPI anchor–deficient cells, the abnormal red cells survive and the proportion can increase to more nearly reflect the size of the abnormal hematopoietic clone (Fig. 10).

Interpretation of Small PNH Clones

As noted above, small PNH clones can be reliably detected in many patients with aplastic anemia and MDS, though the prognostic value of finding a small PNH clone in these disorders remains controversial. In aplastic anemia, some but not all studies have shown that the presence of PNH clones is associated with favorable response to immunosuppressive therapy (51,53–55,63). This conclusion has also been reached in RCUD (53), though for very small (<0.1%) clones, this is based on a study of very few patients. Moreover, the difficulty of distinguishing with certainty hypoplastic MDS from aplastic anemia should be noted. Although patients with these small clones should be followed because of the risk of developing hemolytic PNH, it is important to recognize that these patients are not candidates for treatment with eculizamab because hemolysis with small clones is very rare. In fact, even in patients with hemolytic anemia, the detection of a small granulocyte clone should not be considered diagnostic of classical hemolytic PNH but should instead trigger an investigation for other causes of hemolysis.

Additional Factors Affecting Interpretation

When patients with MDS are screened for PNH clones, the presence of hypogranular neutrophils in peripheral blood can potentially cause difficulties with

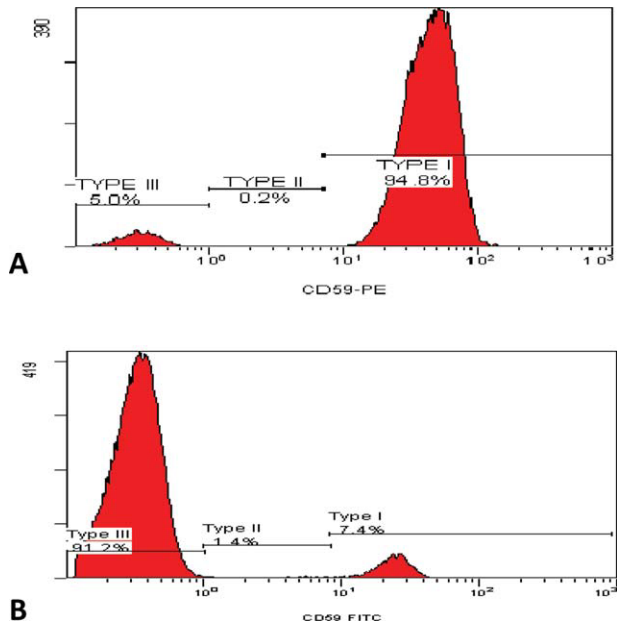

FIG. 10. PNH red cell analysis in a patient with PNH treated with eculizumab. **A**: Prior to therapy the patient had a 95% WBC clone (not shown) but only a 5% RBC clone because of hemolysis and transfusion. **B**: Following successful therapy with eculizumab, the patient's red cell clone matched the white cell clone because his Type III cells were protected from hemolysis (**B**) (CD59 clone MEM-43 from Invitrogen).

gating procedures. Although the altered light scatter characteristics of hypogranular neutrophils often result in significant overlap with monocytes, incorporation of additional, non-GPI-linked markers typically permits clear separation. Hypogranular neutrophils can be found in known PNH patients who are developing MDS, or in de novo MDS patients. Similarly, granulocytes from aged samples (>48-h old) may show altered FSC/SSC scatter characteristics due to poor viability and can also show increased nonspecific binding of antibodies. Another potential pitfall with granulocyte analysis is the presence of immature forms that have significantly weaker expression of some GPI-linked antigens when compared with more mature forms (25,28). In patients with granulocytopenia and relatively high proportions of eosinophils, it may be difficult to construct a pure granulocyte gate unless appropriate markers are used in multicolor analysis.

Reporting Results

In patients with normal expression of GPI-linked antigens, results can be reported relatively easily using language such as: "granulocytes, monocytes, and RBCs show normal expression of GPI-linked antigens. No PNH clones detected." It is extremely important to avoid the use of negative or positive terminology in test reporting to avoid confusion (e.g., "the PNH test was negative" or "all the cells are positive"). Whether further clinical comments are made at this stage depends on the quality of clinical information received with the initial request. If no clinical information is supplied and the test result is normal, then simply providing some indication of the sensitivity of the assay should suffice. If, however, there is good supporting clinical evidence that the patient has aplastic anemia or myelodysplastic syndrome, then repeat testing may be recommended in the report. In contrast, there is no requirement to suggest repeat testing for patients with unexplained thrombosis, hemolysis, or hemoglobinuria who have no detectable PNH clones.

If an abnormality is detected, it is important to communicate this result clearly and promptly, especially when the findings are compatible with a new diagnosis of classical PNH. The proportion of abnormal cells (i.e., the size of the PNH clone) in each lineage tested should be reported, including information on Type II cells if present, without overly complex numerical description of individual antibody results. As noted above, if only a small clone is detected, it is preferable to include language that makes clear that this is not equivalent to a diagnosis of hemolytic PNH. Care should also be taken not to overinterpret the significance of very small clones in a routine clinical report, though it is very important that these be communicated in research studies.

In patients with an established diagnosis of PNH, repeat testing may be performed for a variety of reasons, including monitoring the response to therapy, investigation of sudden changes in blood count parameters, increased transfusion requirement, and evaluation of clone size over time. With each report, in addition to reporting quantitative results on the current sample, comments should be made as to how the current results compare with previous results, and the possible clinical significance of such changes discussed.

Examples of laboratory reports are shown in Appendix B.

SUMMARY AND FUTURE DIRECTIONS

Flow cytometry is now widely accepted as the method of choice for diagnosing hemolytic PNH, and for detecting GPI-anchor protein-deficient clones in subclinical PNH and related bone marrow disorders. In this document, we have attempted to present a consensus view of practitioners with considerable experience in testing samples from PNH patients and to provide guidelines that should be helpful both to laboratories interested in beginning PNH testing and to those seeking to improve their existing PNH testing. The authors recognize, however, that this is a work in progress. Among others, questions that future research should address include the following: (1) Under what circumstances is it necessary to perform PNH testing in patients with otherwise typical myelodysplastic or myeloproliferative bone marrow disorders? (2) Is there

a single specific combination of markers that should be used for gating and analysis to the exclusion of others? (3) Even if this degree of standardization cannot be achieved, should FLAER be mandated as an essential reagent in all combinations used to analyze PNH clones on leukocytes? (4) What marker is better than CD14 to accompany FLAER in analysis of PNH clones on monocytes? Are there additional granulocyte markers that are better than CD24? Is there a single marker (in addition to FLAER) that can adequately identify both granulocytes and monocytes? (5) Can a simple, reproducible high sensitivity screening red cell assay be recommended such that a negative result excludes the presence of PNH clones, and what would be the best combination of markers to use in such an assay? (6) What limits can be put on the interlaboratory reproducibility of high sensitivity assays? and (7) Is there additional important clinical information that can be derived from more detailed analysis of data, as for example by studying patients with Type II granulocytes, or those in whom the size of monocyte and granulocyte clones differs?

Even though there are still questions to be answered, the authors believe that by following the guidelines suggested here, it should be possible for the great majority of laboratories to provide the testing needed for routine diagnosis of PNH. The higher-sensitivity methods described here are more challenging, there is less consensus about best methodology, and the need for performing them is more controversial. However, as we continue to learn about the clinical significance of small PNH clones in a variety of disorders, it is hoped that these guidelines will help more laboratories to perform these tests accurately.

ACKNOWLEDGMENTS

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APPENDIX A: ANTIBODY COMBINATIONS FOR DETECTING PNH WHITE BLOOD CELL CLONES

		Colors					
	Cells	1	2	3	4	5	6
3 color	Grans	**FLAER**	**CD24**	CD15			
3 color	Monos	**FLAER**	**CD14**	CD33			
4 color	Grans	**FLAER**	**CD24**	CD15	CD45		
4 color	Monos	**FLAER**	**CD14**	CD33	CD45		
4 color	G+M	**FLAER**	**CD24**	**CD14**	CD33		
5 color*	G+M	**FLAER**	**CD24**	**CD14**	CD15/ 33	CD45	
5 color	G+M	**FLAER**	**CD24**	**CD14**	CD15	CD33	
6 color	G+M	**FLAER**	**CD24**	**CD14**	CD15	CD33	CD45

Markers shown in Bold are used to detect GPI-anchor deficient cells, while those shown in normal font are used for gating. Exact choice of fluorochromes (eg perCP Cy 5.5 vs. PE-TR for ''color 3''), can vary. Also, fluorochromes for some reagents, especially gating reagents, may be switched. The five and six color combinations, and some of the four color combinations (e.g. FLAER/CD24/CD15/CD45) shown here will work well for high-sensitivity analysis.

*In this five-color combination, two tubes are necessary to detect both granulocytes and monocytes optimally; the combination of CD45, CD15, and SSC identifies granulocytes and can often isolate monocytes, but optimal identification of monocytes requires a second tube gated using CD33.

APPENDIX B: SAMPLE REPORTS

Accession #:
Accession Date:
Collection Date:
Attending Physician:
Procedure Physician/Copies To:

Outside Institution:

Patient Name:
MRN:
Location:
DOB/Age/Sex: **Age: 32 F**
Account #:
Specimen Class: **Special Procedure Only**

PATIENT HISTORY:
Inflammatory bowel disease with pancytopenia for past 4 months. Bone marrow aspirate and biopsy showed hypocellularity. Peripheral blood submitted for flow cytometric evaluation for PNH.

SPECIMEN(S) RECEIVED:
Peripheral Blood for Flow Cytometry

INTERPRETATION
Flow cytometric immunophenotypic studies performed on peripheral blood demonstrate decreased expression of the GPI-linked antigens CD55 and CD59 on a population of red blood cells, decreased expression of the GPI-linked antigen CD55, and weak intensity of the GPI-linked antigen CD59 by a population of white blood cells (monocytes and neutrophils), and slightly decreased binding of Aerolysin to a population of white blood cells (monocytes and neutrophils). These results indicate the presence of populations of cells with a PNH-type phenotype and are similar to the previous analysis.

Cells with a PNH-type phenotype represent approximately 18 - 20% of neutrophils and approximately 2% of red blood cells.

A PNH-type phenotype has been described in a variety of clinical settings and therefore, these flow cytometric results should be correlated with the clinical and laboratory information.[1] In classic PNH, the PNH-type population usually represents > 10% of cells at presentation and is accompanied by intravascular hemolysis. A PNH-type granulocyte population > 50% has been reported to be associated with a significant risk of thrombosis.[2] A subset of patients with a significant PNH-type population and intra-vascular hemolysis have another underlying bone marrow disorder such as aplastic anemia (AA) or a myelodysplastic syndrome (MDS). Small populations of PNH-type cells (less than 10%) have been reported in AA and MDS in the absence of hemolysis (subclinical PNH), and even when the PNH-type population is very small (< 1%) have been reported to be associated with response to immunosuppressive therapy.[3, 4]

References:
1. Diagnosis and management of PNH. Parker et al., *Blood* 2005;106:3699–3709.
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3. Minor population of CD55(-) CD59(-) blood cells predicts response to immunosuppressive therapy and prognosis in patients with aplastic anemia. Sugimori C et al., *Blood* 2006;107:1308-1314.

Pathologist:
** Report Electronically Signed Out **
Pathologist:

My electronic signature on this report is attestation that I have personally reviewed the submitted material and/or data included in this special procedure report and that my interpretation reflects this evaluation.

FLOW CYTOMETRY REPORT – PNH EVALUATION

SAMPLE REPORT

Name:	PNH, Negative	Pathology Number:	F-07-10349
DOB:	11/09/1956 Sex: M MR #: 987654321	Date of Procedure:	3/10/2007
Facility:	Ordering Facility	Date of Accession:	3/10/2007
Dept:	Outpatient		

Physician: Ordering Provider, M.D.
Ordering Facility
Street Name
City, State Zip code
(999) 123-4567

Copies to: Other Providers/clinicians

TISSUE/SPECIMEN: Peripheral Blood in Heparin

DIAGNOSIS: NO PHENOTYPIC EVIDENCE OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA (PNH)

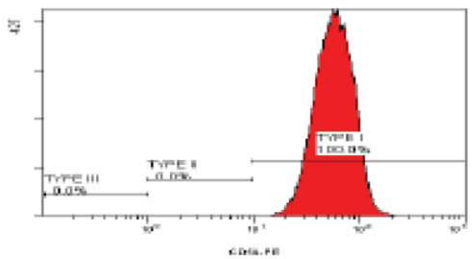
Comment: Flow cytometric analysis does not show any evidence of a PNH clone based upon analysis of a variety of GPI-linked antibodies on red blood cells, monocytes and granulocytes. These findings do not support a diagnosis of PNH. Clinical correlation is recommended.

Flow Results: ***Immunophenotypic analysis*** was performed using gating antibodies CD45, CD15, CD33, CD64, GPI-linked antibodies CD59, CD14, CD24, as well as fluorescent Aerolysin (FLAER).

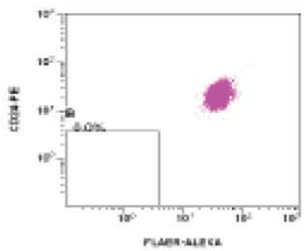
Red Blood Cells: No evidence of decreased or absent CD59 expression

Monocytes: No evidence of decreased or absent expression of FLAER or CD14

Granulocytes: No evidence of decreased or absent expression of FLAER or CD24



Sample histogram of a typical patient with no evidence of PNH in RBC's



Sample histogram of a typical patient with no evidence of PNH in Granulocytes

The markers used for this flow cytometric analysis are labeled as Analyte Specific Reagents (ASR) and are used for clinical purposes. The performance characteristics of these markers have been determined by DCDS-Flow Cytometry Laboratory. Their use has not been approved by the U.S. Food and Drug Administration; the FDA has determined that such approval is not necessary.

Electronic Signature
Pathologist/Technologist
Date

FLOW CYTOMETRY REPORT – PNH EVALUATION

SAMPLE REPORT

Name: PNH, Positive
DOB: 7/31/1973 Sex: M MR #: 123456789
Facility: Ordering Facility
Dept: Outpatient

Pathology Number: F-07-20349
Date of Procedure: 6/15/2007
Date of Accession: 6/15/2007

Physician: Ordering Provider, M.D.
Ordering Facility
Street Name
City, State Zip code
(999) 123-4567

Copies to: Other providers/clinicians

TISSUE/SPECIMEN: Peripheral Blood in Heparin

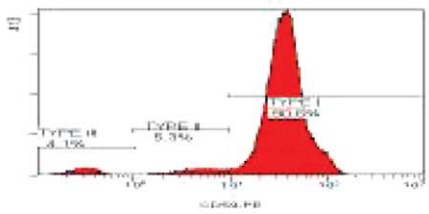
DIAGNOSIS: PNH CLONE IDENTIFIED IN BOTH WBC AND RBC

Comment: Flow cytometric analysis shows a PNH clone within the granulocytes (60.9%), monocytes (61.5%) and RBC's (9.4%). These findings are consistent with a diagnosis of paroxysmal nocturnal hemoglobinuria (PNH). Any potential difference in clone size between the white blood cells and the red blood cells may be due to hemolysis and/or recent transfusion. The PNH clone in the monocytes and granulocytes showed a bimodal distribution, indicating Type II and Type III cells. The clinical significance of this finding is still under investigation.

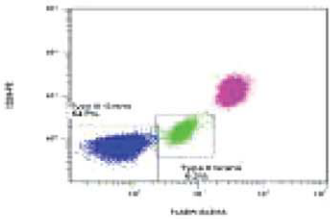
Reference: Richards et al: Diagnosis and Management of PNH, Blood 2005, 106 (12)

Flow Results: ***Immunophenotypic analysis*** was performed using gating antibodies CD45, CD15, CD33, CD64, GPI-linked antibodies CD59, CD14, CD24, as well as fluorescent Aerolysin (FLAER).

Cell Type	Deficiency	Result
RBC	Type II (partial CD59 deficiency)	5.3%
	Type III (complete CD59 deficiency)	4.1%
	PNH Clone size (Type II and Type III combined)	9.4%
WBC - Monocytes	FLAER/CD14 Deficiency	61.5% (57.2% Type III + 4.3% Type II)
WBC - Granulocytes	FLAER/CD24 Deficiency	60.9 (54.7% Type III + 6.2% Type II)



Type III and Type II PNH clone in RBC's



Type III (blue) and Type II (green) PNH Clone in Granulocytes

The markers used for this flow cytometric analysis are labeled as Analyte Specific Reagents (ASR) and are used for clinical purposes. The performance characteristics of these markers have been determined by DCDS-Flow Cytometry Laboratory. Their use has not been approved by the U.S. Food and Drug Administration; the FDA has determined that such approval is not necessary.

Electronic Signature
Pathologist/Technologist
Date

Positive PNH

Page 1 of 1